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CMKLR1 Antagonist Alpha-NETA Protects against Diabetic Nephropathy in Mice

Zining Peng[a] Xueyi Wang[a] Qing Zhu[a] Huili Wang[a] Bing Li[a]
Xinxin Pang[b] Jiarui Han[a]

[a]Second clinical medical College, Henan University of Chinese Medicine, Zhengzhou, China; [b]Department of Nephropathy, Henan Provincial Hospital of Traditional Chinese Medicine (The Second Hospital Affiliated to Henan University of Chinese Medicine), Zhengzhou, China

Keywords
Diabetic neuropathy · Chemokine-like receptor 1 · 2-(Anaphthoyl)ethyltrimethylammonium iodide · Chemerin · Inflammation

Abstract
Introduction: Diabetic nephropathy (DN) is a common complication in diabetic patients. Chemerin, a novel adipokine, has been associated with renal damage in DN. The chemerin chemokine-like receptor 1 (CMKLR1) has been reported to participate in DN. In this study, we aimed to investigate the effect of a CMKLR1 antagonist, 2-(anaphthoyl)ethyltrimethylammonium iodide (α-NETA), on DN. ***Methods:*** To induce diabetes, 8-week-old male C57BL/6J mice were given a single intraperitoneal injection of 65 mg/kg streptozotocin (STZ). Diabetic mice were randomly assigned to receive daily doses of 0, 5, or 10 mg/kg α-NETA for 4 weeks. ***Results:*** α-NETA dose-dependently induced body weight and reduced fasting blood glucose levels in STZ-induced diabetic mice. Furthermore, α-NETA significantly reduced the expressions of renal injury markers, including serum creatinine, kidney weight/body weight, urine volume, total proteins, and albumin in the urine, and increased creatinine clearance. Periodic acid-Schiff staining also indicated that α-NETA could effectively ameliorate renal injuries in DN mice. In addition, α-NETA inhibited renal inflammation and the expressions of chemerin and CMKLR1 in mice with DN. ***Conclusion:*** In summary, our findings suggested that α-NETA has beneficial effects on the management of DN. Specifically, α-NETA effectively ameliorated renal damage and inflammation in a dose-dependent manner in mice with DN. Thus, targeting the chemerin and CMKLR1 axis with α-NETA may be a promising therapeutic strategy for the treatment of DN.

Introduction

Diabetic nephropathy (DN) is a critical complication of diabetes mellitus, characterized by the progressive reduction of proteinuria and glomerular filtration rate over time [1]. As a diabetic microvascular complication, DN is becoming increasingly prevalent worldwide, particularly in developed countries [2, 3]. Because of the metabolic complexity of DN, early prevention is crucial for successful management before it progresses to end-

karger@karger.com
www.karger.com/kbr

Correspondence to:
Jiarui Han, hanjr@hactcm.edu.cn

stage renal disease. Thus, there is a growing need to identify effective strategies to prevent or slow the progression of DN in diabetic patients.

Chemerin is considered a chemoattractant adipokine, playing a crucial role in adipocyte differentiation and glucose uptake [4]. Previous studies have suggested that serum levels of chemerin are elevated in the patients with type 2 diabetes mellitus (T2DM) and renal damage, with a negative correlation observed between chemerin levels and renal function [5–8]. In addition, in the models of hypertensive nephropathy and glomerulonephritis, renal chemerin expression was found to be markedly upregulated, exhibiting a positive correlation with fibrotic and inflammatory markers [9]. Chemerin chemokine-like receptor 1 (CMKLR1) is the endogenous ligand of chemerin. Upon binding to chemerin, the receptor promotes the release of calcium ions within cells, subsequently activating nuclear transcription factor (NF-κB) and cellular signaling pathways, including extracellular signal-regulated kinase 1 (ERK-1). Through these mechanisms, CMKLR1 plays a significant role in the pathogenesis of numerous diseases, including cardiovascular disease, metabolic disorders, and inflammatory responses. Chemerin/CMKLR1 axis was reported to accelerate the inflammatory responses of glomerular endothelial cells in DN [10]. Furthermore, the CMKLR1 antagonist CCX832 was found to attenuate renal reactive oxygen species production in diabetic mice [10]. 2-(Anaphthoyl)ethyltrimethylammonium iodide (α-NETA), a small molecule antagonist of CMKLR1, has been found to significantly inhibit central nervous system autoimmune inflammatory disease, such as multiple sclerosis, which is achieved through the suppression of chemerin-triggered β-arrestin2 association with CMKLR1, as well as CMKLR1+ cell migration [11]. Given the indications that the novel CMKLR1 antagonist may be beneficial in the management of DN, we aimed to investigate the nephroprotective effect of α-NETA in diabetic mice in this study.

Materials and Methods

DN Mouse Model

To induce diabetes, a single dose of streptozotocin (STZ, 65 mg/kg, dissolved in sodium citrate buffer [0.1 M, pH 4.5]) was intraperitoneally administrated to 8-week-old C57BL/6J male mice. Three days later, diabetes was confirmed by measuring fasting blood glucose levels through tail vein using a glucometer. Mice with fasting blood glucose over 250 mg/dL were identified as diabetic and randomly divided into three groups. The mice were orally administrated α-NETA (0, 5, or 10 mg/kg, N275326, Aladdin) daily for 4 weeks: STZ, STZ+α-NETA5, and STZ+α-NETA. Age-matched C57BL/6J male mice were used as healthy controls and were injected with 0.1 M sodium citrate buffer. The mice were then randomly divided into two groups and orally administered 10 mg/kg of α-NETA (α-NETA group) or vehicle (control group). The study was approved by the Ethics Committee of the Henan University of Chinese Medicine.

Periodic Acid-Schiff Staining

Paraffin-embedded kidney blocks were cut into 4 μm-thick sections, then stained with periodic acid-Schiff as previously described [12]. Stromal hyperplasia in the glomerular mesangial area and basement membrane thickening in the glomerular capillary plexus were assessed to evaluate glomerular damage. The extent of glomerulosclerosis was graded using a semi-quantitative scoring method based on the proportion of sclerotic area: grade 0 for normal glomeruli, grade 1 for minimal sclerosis (up to 1/4), grade 2 for moderate sclerosis (1/4 to 1/2), grade 3 for moderate-severe sclerosis (1/2 to 3/4), and grade 4 for severe sclerosis (3/4 to 4/4). The glomerulosclerotic index (GI) score was analyzed using the following formula: GI score = $(1 \times N1) + (2 \times N2) + (3 \times N3) + (4 \times N4)/N0 + N1 + N2 + N3 + N4$, where Nx is the number of glomeruli in each grade of glomerulosclerosis. This analysis was performed with the observer blinded to the treatment. 60 glomeruli per mouse were analyzed.

ELISA

Renal tissue samples were collected from mice and homogenized in a lysis buffer containing protease inhibitors to prevent degradation of the cytokines. Then, the homogenate was centrifuged at high speed to pellet any cellular debris, and then the supernatant was transferred to a new tube for analysis. The inflammatory cytokine secretions in the serum and renal tissues were determined using ELISA kits according to the manufacturer's instruction (R&D Biosystem): IL-6 (M6000B), TNF-α (MTA00B), IL-8 (DY442), chemerin (MCHM00). CMKLR1 (E03C1829) was purchased from Bluegene (Shanghai, China).

qRT-PCR

Kidney tissue was collected from mice and immediately placed in the TRIzol buffer. mRNA was extracted using RNeasy Kit (Qiagen, Germany). Then mRNA was transcripted into cDNA using iScript cDNA Synthesis Kit (Bio-Rad; Hercules, CA, USA). qRT-PCR was performed as previously described [13]. *GAPDH* was used as an internal control. The sequences of primers are listed below.

- *IL-6* F, TAGTCCTTCCTACCCCAATTTCC,
  - R, TTGGTCCTTAGCCACTCCTTC′
- TNF-α: F, GGAACACGTCGTGGGATAATG,
  - R, GGCAGACTTTGGATGCTTCTT;
- *IL-8*: F, TCGAGACCATTTACTGCAACAG,
  - R, CATTGCCGGTGGAAATTCCTT;
- Chemerin: F, GGAGATCGGTGTGGACAGTG,
  - R, GGGTCCAGTTTGATGCAGG;
- CMKLR1 F, ATGGAGTACGACGCTTACAACG,
  - R, GGTGGCGATGACAATCACCA;
- GAPDH: F, TGCATCCTGCACCACCAACTGC,
  - R, ACAGCCTTGGCAGCACCAGTGG.

Western Blot

Renal tissue samples were collected from mice and lysed in radioimmunoprecipitation assay buffer with proteinase inhibitor to inhibit protein degradation. The homogenate was centrifuged at 12,000 *g* for 15 min to get supernatant. Protein concentration was determined using BCA kit. The protein sample was mixed with a loading buffer that contains a reducing agent to denature the proteins and a tracking dye to monitor the migration of the proteins during electrophoresis. The protein samples were loaded onto a polyacrylamide gel for electrophoresis, along with molecular weight markers to determine the size of the proteins. Western blot was performed as previously described [14]. Antibodies were used against chemerin (1:2,000, ab103153), CMKLR1 (1:1,000, ab230442), and GAPDH (1:3,000, ab8245). All antibodies were purchased from Abcam.

Statistical Analysis

Data were analyzed using GraphPad and presented as means ± SD. The different groups were compared using a one-way ANOVA analysis followed by Dunnett's T3 multiple comparisons test. $*p < 0.05$ was regarded as a significant difference.

Results

A-NETA *Dose-Dependently Improved Renal Function in Diabetic Mice*

After 4 weeks of treatment, α-NETA had no significant effect on fasting blood glucose and body weight in healthy mice (Fig. 1a, b), indicating its safety. In contrast, diabetic mice exhibited classic symptoms of diabetes, including high fasting blood glucose levels and weight loss, which were dose-dependently improved by α-NETA (Fig. 1a, b). Furthermore, diabetic mice showed severe renal dysfunction, as evidenced by increased kidney weight/body weight ratios and elevated serum creatinine levels (Fig. 1c, d), both of which were reduced in a dose-dependent manner after α-NETA treatment. These results suggest that α-NETA has a protective effect on renal function in diabetic mice.

A-NETA *Dose-Dependently Improved Kidney Injury in STZ-Induced Diabetic Mice*

Next, we evaluated the protective effect of α-NETA on kidney injury in diabetic mice. Compared to healthy mice, diabetic mice displayed severe kidney injury, including increased urine volume, urine total protein, urine albumin, and decreased creatinine clearance, which were all restored by α-NETA in a dose-dependent manner (Fig. 2a–d). In addition, α-NETA did not alter renal function in healthy mice. Furthermore, periodic acid-Schiff staining also indicated that α-NETA could effectively restore kidney damage in diabetic mice (Fig. 3). Collectively, these data suggest that α-NETA exerts a protective effect on kidney function in diabetic mice.

A-NETA *Dose-Dependently Reduced Renal Inflammation in STZ-Induced Diabetic Mice*

Furthermore, we explored the anti-inflammatory effect of α-NETA in diabetic mice. Diabetic mice displayed an activated inflammatory response in comparison with control mice. Both the secreted cytokines in the serum and mRNA levels in the kidney, including IL-6, TNF-α, and IL-8, were significantly induced in diabetic mice (Fig. 4a–f). α-NETA dose-dependently reduced the increased protein and mRNA levels of these cytokines. These results indicate that α-NETA has an anti-inflammatory effect in diabetic mice.

A-NETA *Inhibited the Chemerin/CMKLR1 Signaling Pathway in STZ-Induced DN Mice*

Interestingly, we found that α-NETA, which acts as an antagonist of CMKLR1, significantly reduced the serum levels of chemerin and CMKLR1 in a dose-dependent way (Fig. 5a, b). We also observed that it significantly inhibited the protein and mRNA levels of chemerin (Fig. 5c, d, f) and CMKLR1 (Fig. 5c, e, g), which were induced in diabetic kidneys.

Discussion

Chemerin is a novel cytokine that was traditionally regarded as an inflammation-produced chemotactic factor but now is believed to be an adipokine regulating adipose metabolism and energy balance [15, 16]. Increasing evidence has demonstrated that circulating chemerin levels were increased in diabetic patients, including those with gestational diabetes mellitus [17, 18], and chemerin was also regarded as a predictor for T2DM [19, 20]. However, the relationship between diabetes and chemerin was controversial. Some studies proved that chemerin accelerated glucose intolerance in diabetes [21] or induced insulin resistance in skeletal muscle cells [22]. Others believed that chemerin contributed to inflammation rather than diabetes [23]. However, chemerin was considered an endocrine link among T2DM, obesity, and inflammation [24, 25]. Thus, targeting chemerin was a promising therapy for the management of obesity and T2DM. Our current study also confirmed that inhibiting chemerin and CMKLR1 by α-NETA could effectively reduce the symptoms of

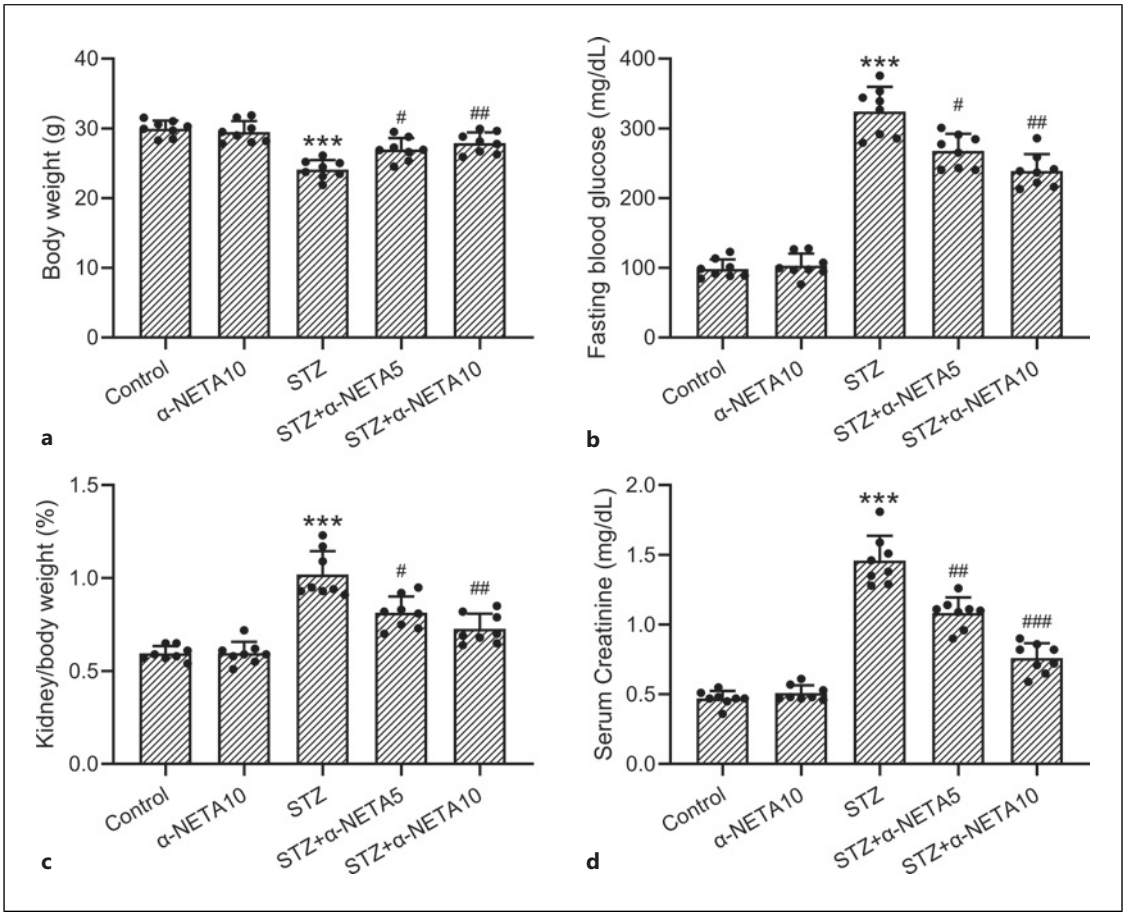

Fig. 1. α-NETA treatment on body weight (**a**), fasting blood glucose levels (**b**), kidney weight/body weight (**c**), and serum creatinine (**d**) of mice model of DN at the end of 4 weeks treatment. Serum levels of creatinine (Cr) were analyzed by an automatic analyzer (cobas® 8000 modular analyzer series; Roche Diagnostics). 8 mice were used for each group. Data are presented as means ± SD. ***$p < 0.001$ compared to control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to STZ.

diabetes as reflected by the decrease in fasting blood glucose and increase in body weight in STZ-injected mice. However, we did not measure the effect of α-NETA on insulin sensitivity and glucose tolerance, which might be our limitation.

Chemerin levels in the serum were proved to be reversely associated with renal function, not only in the general population [5] but also in incident dialysis patients [26]. Moreover, chemerin levels were also elevated in T2DM patients with macroalbuminuria [27]. Chemerin/CMKLR1 axis was reported to promote DN [28]. Therefore, suppressing the chemerin/CMKLR1 axis could be a promising therapy to treat DN. For instance, rosiglitazone was proven to effectively ameliorate DN by inhibiting the expressions of chemerin and CMKLR1 in diabetic mice [29]. Therefore, as a small molecule antagonist of CMKLR1, α-NETA is proposed to also effectively ameliorate DN in the same mouse model of DN.

Systemic and local microinflammation are characteristics of DN. Accumulating studies ranging from epidemiological studies to in vivo experiments proved that overexpressed inflammatory cytokines accelerated renal fibrosis in different glomerular nephritis, eventually contributing to renal dysfunction and renal failure [30]. Therefore, targeting inflammatory cytokines was considered a promising therapeutic strategy for the management of DN. For example, suppressing monocyte chemoattractant protein-1 effectively decreased urinary protein in DN [31]. Previous publications have revealed that chemerin secretion was correlated with the secretions of some inflammatory cytokines,

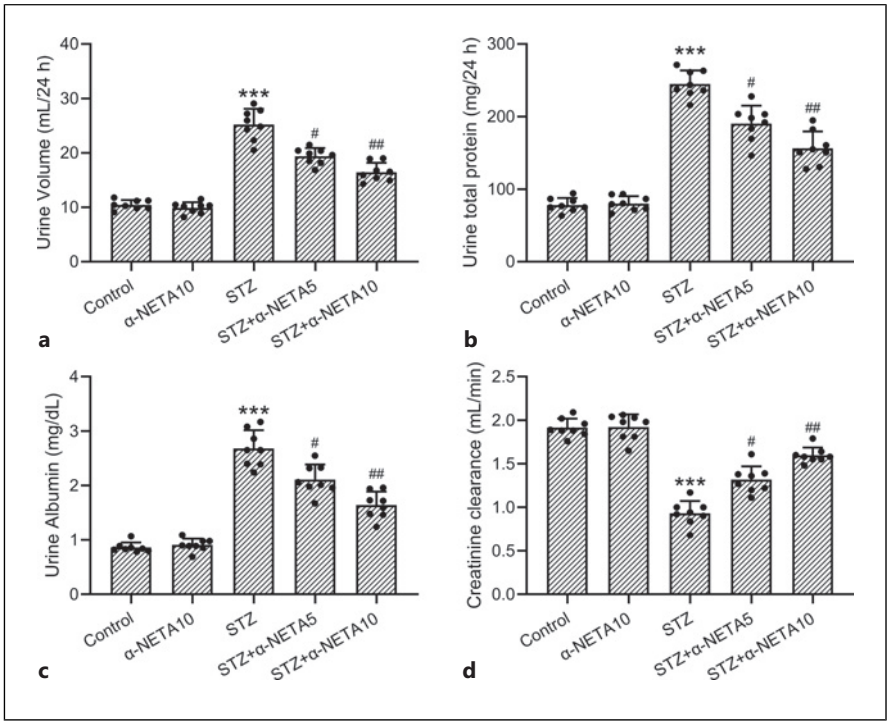

Fig. 2. Effects of α-NETA treatment on urine volume (**a**), urine total proteins (**b**), urine albumin (**c**), and creatinine clearance (**d**) in urine of DN mice at the end of 4 weeks of treatment. Urinary levels of albumin, total proteins, and creatinine were analyzed by an automatic analyzer (cobas® 8000 modular analyzer series; Roche Diagnostics). 8 mice were used for each group.

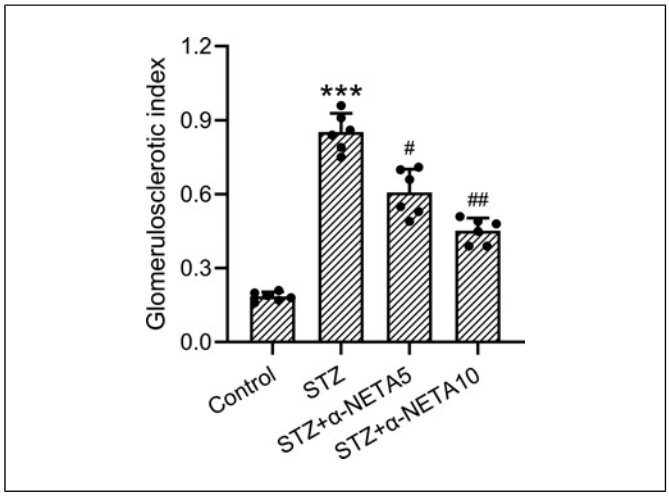

Fig. 3. Effects of α-NETA treatment on histopathological change of the kidney at the end of 4 weeks treatment. Glomerulosclerotic index was quantified based on PAS staining. The data were obtained from 6 mice in each group and the data point indicates the average of single mouse from 60 glomeruli. PAS, periodic acid-Schiff.

including C-reactive protein, TNF-α, and IL-6 [32]. Moreover, the chemerin/CMKLR1 signaling activated inflammation of glomerular endothelial cells in DN [28]; thus, these studies indicated that the chemerin/CMKLR1 axis serves as a functional link between inflammation and DN and inhibiting this axis might be a promising approach to manage DN by suppressing inflammatory responses. This is consistent with our current findings that as a small molecule antagonist of CMKLR1, α-NETA attenuated inflammatory responses in STZ-induced DN mice by inhibiting the expressions of chemerin and CMKLR1.

It is worth noting that there are three chemerin receptors, including CMKLR1, CCRL2, and GPR1, which have been documented [33]. While the functions of the last two receptors (CCRL2 and GPR1) are still unclear, CMKLR1 is a well-defined G-protein-coupled receptor that has been implicated in DN.

We observed that α-NETA reduced blood glucose levels in DN mice. This is our limitation that we did not explore its mechanism. However, previous study demonstrated that CCX832, another antagonist of CMKLR1, reduced plasma glucose and insulin levels in db/db mice, as well as HOMA index, which is a measure of insulin resistance [34]. This suggests that blocking the activity of CMKLR1 may improve insulin sensitivity and glucose homeostasis. Another study also indicated that mice lacking CMKLR1 receptors fed with a high-fat diet had reduced blood glucose and serum insulin levels, suggesting blocking CMKLR1 activity may improve

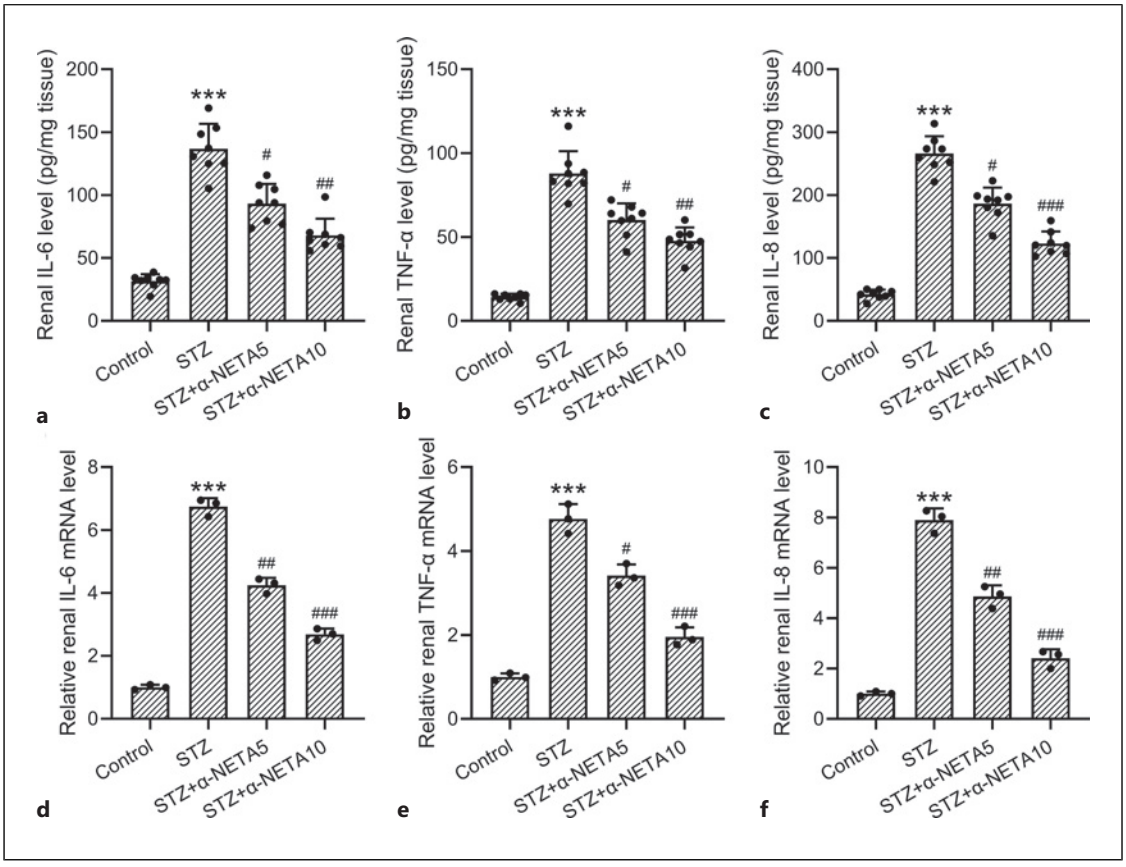

Fig. 4. Effects of α-NETA treatment on renal inflammatory responses at the end of 4 weeks treatment. The concentrations of IL-6 (**a**), TNF-α (**b**), and IL-8 (**c**) in renal tissues were measured by ELISA. 8 mice were used for each group. RT-qPCR was used to measure the mRNA expressions of IL-6 (**d**), TNF-α (**e**), and IL-8 (**f**) in renal tissues. The data were obtained from 3 repeated experiments using renal homogenate from each group including 8 mice.

glucose metabolism [35]. Taken together, these findings suggest that blocking the activity of CMKLR1 using antagonist like α-NETA may reduce blood glucose levels and improve insulin sensitivity. But further studies are needed to fully understand the mechanisms by which CMKLR1 and its antagonists affect glucose homeostasis and insulin secretion.

Studies have shown that the level of CMKLR1 is significantly elevated in the serum of patients with DN, likely due to the secretions of transforming growth factor-β [28]. This receptor also plays a role in renal damage in DN mice [29]. Our findings were consistent with the previous studies that have reported significant elevation of CMKLR1 levels in mice with DN. Given the well-established role of CMKLR1 in DN and the unclear function of the other two chemerin receptors, CCRL2 and GPR1, our study only focused on the small molecule antagonist of CMKLR1 in DN development. However, future research should investigate the potential roles of CCRL2 and GPR1 in DN, which would provide additional insights into the mechanisms of this disease.

However, it is not clear whether this effect is a direct result of α-NETA's action on the kidney or an indirect result of α-NETA's ability to correct metabolic abnormalities that contribute to nephropathy. It is important to acknowledge this limitation of the study as it is difficult to determine the exact mechanism by which α-NETA is providing protection against DN. Further studies are needed to explore the potential direct effects of α-NETA on the kidney and to better understand the metabolic abnormalities that contribute to nephropathy in diabetes. It is also possible that α-NETA is having both direct and indirect effects on the kidney

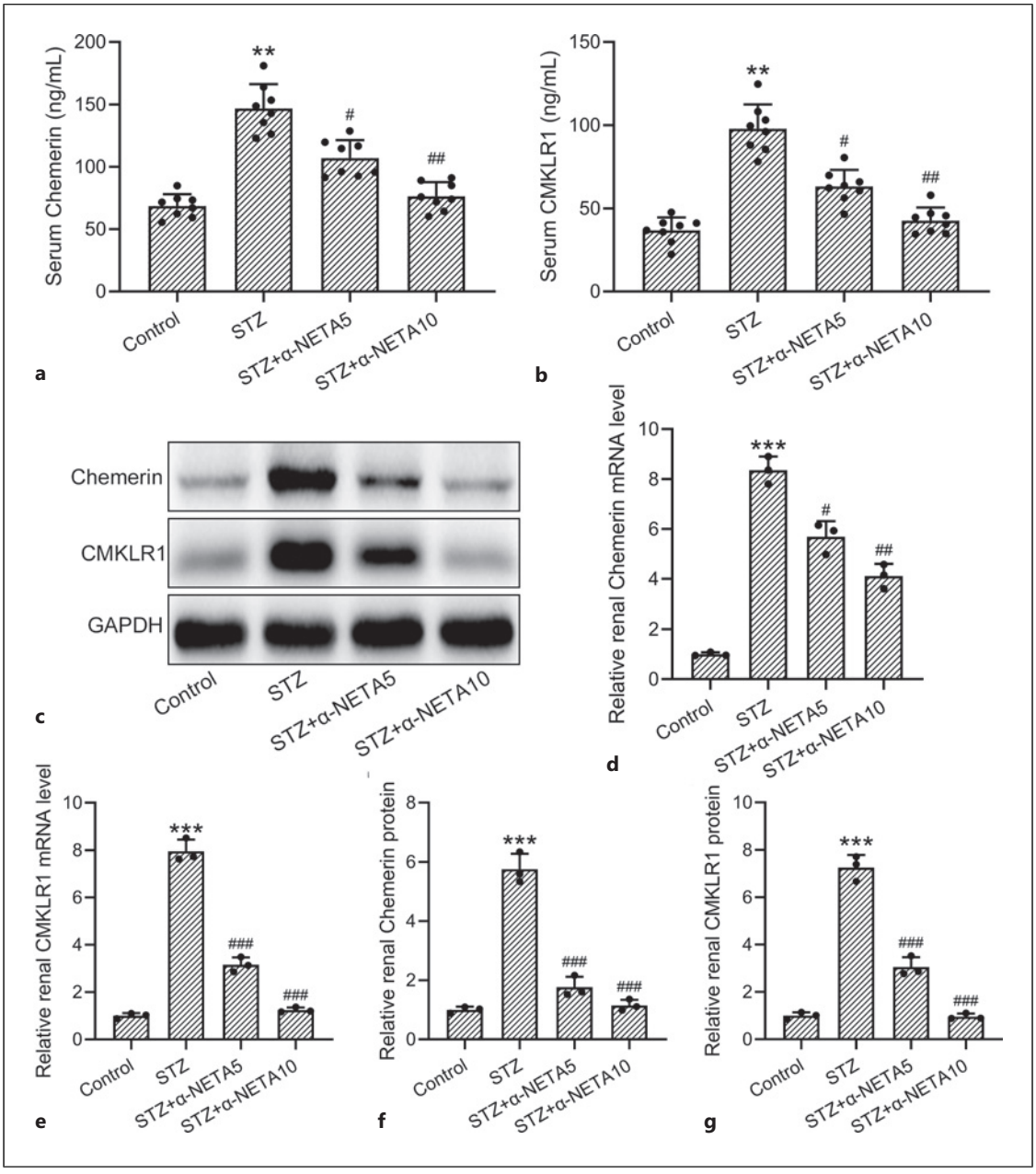

Fig. 5. Effects of α-NETA treatment on renal chemerin/CMKLR1 signaling pathway in mice model of DN. Serum levels of chemerin (**a**) and CMKLR1 (**b**) were measure by ELISA at the end of 4 weeks of treatment. 8 mice were used for each group. RT-qPCR was used to measure the mRNA expressions of chemerin (**d**) and CMKLR1 (**e**) in renal tissues at the end of 4 weeks of treatment. Western blot was used to measure the protein expressions of chemerin and CMKLR1 (**c**) and the relative expressions were normalized to control (**f**, **g**). The data were obtained from 3 repeated experiments using renal homogenate from each group including 8 mice.

and metabolic abnormalities and that these effects are interrelated. Nevertheless, it is important for researchers to clarify the mechanism of action of α-NETA to better understand its potential therapeutic benefits for DN.

Conclusion

As a small molecule antagonist of CMKLR1, our study demonstrated that α-NETA effectively ameliorated renal damage and inflammation in a dose-dependent manner

in mice with DN. These findings suggested that α-NETA has a potential therapeutic effect for the management of DN.

Statement of Ethics

The study was approved by the Ethics Committee of the Henan University of Chinese Medicine (JHU-2978). This study was performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85-23 Rev. 1985).

Conflict of Interest Statement

The authors have no conflict of interest to declare.

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Author Contributions

Concept or design, acquisition of data, and analysis or interpretation of data: Zining Peng, Xueyi Wang, Xinxin Pang, and Jiarui Han. Drafting of the manuscript: Zining Peng, Xueyi Wang, Qing Zhu, Huili Wang, Bing Li, Xinxin Pang, and Jiarui Han. Critical revision of the manuscript for important intellectual content: all authors. All authors had full access to the data, contributed to the study, approved the final version for publication, and take responsibility for its accuracy and integrity.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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